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AUTHORITY

AMXFD ltr, 9 Feb 1972

TRANSLATION NO. 1193

DATE: 24 Aug 1964

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

THE REACTION OF BACTERIA TO SOME HEMOGLOBIN DERIVATIVES

/Following is translation of a German-language article by Dr. Hugo Kammerer, Clinico-medical Institute of Munich University, in Proceedings of the 31st Congress of the German Society for Internal Medicine, 1914, pp 704-709./

My investigations, undertaken in collaboration with Hans Fischer (Munich), were intended initially to answer the question whether and how hemoglobin and its derivatives can be changed and decomposed by bacteria, a question Fischer had undertaken to answer once before. In addition to human blood, the pure derivatives -- heme, mesohemin, hematoporphyrin, mesoporphyrin and bilirubin -- were added to various nutrient media -- exercising the necessary care to maintain the slight alkaline reaction -- initially in average concentrations of the pigments of 1:1000 to 1:2000. The susceptibility to growth of the bacteria was tested on control culture media. Our one original question then immediately became two because we needed to determine, first, how the bacteria react to the hemoglobin derivatives and, second, how the hemoglobin derivatives react to the bacteria. Because of the brief time available, I cannot here enter on our initial question and these experiments, moreover, have not yet been terminated. However, I should like to stress one fact: the incubation of pure cultures of a great variety of species of bacteria on solid and liquid nutrient media with the addition of heme, mesohemin, hematoporphyrin, and bilirubin (mesoporphyrin always flocculated and therefore could not be utilized) never showed any intensive change of the pigments but always a pronounced sedimentation of these pigments in the colony. Although a few species of bacteria were able to here and there change the color tone, this is probably mainly due to the acid or akaline reaction of the bacteria.

My interest became engaged far more in the effect of the hemoglobin derivatives on the bacteria. The agar plate cultures utilized contained heme, mesohemin, hematoporphyrin and bilirubin in a concentration of 1:2000. I soon noticed that a part of the bacteria investigated always

developed very well on the heme, hematoporphyrin and bilirubin cultures but not on the mesoporphyrin plates. I found that it was always the members of certain very definite groups of species of bacteria which here showed the same behavior as indicated in the table below.

Action of Mesohemin On Bacteria

-	Gram	+	Gram
B. pyocyaneus	-	Staphyloc. aureus	+
B. fluorescens	-	Staphyloc. albus	+
B. prodigiosus	-	Staphyloc. citreus	+
V. Metschnikoff	-	Micr. tetragenus	+
V. proteus	-	Sarcine	+
B. typhi	-	Streptokokkus	+
B. paratyphi	-	Pneumokokkus	+
B. enteritidis G.	-	B. anthracis	+
B. suipestifer	-	B. Megatherium	+
B. coli	-	B. mycoides	+
B. lactis	-	B. subtilis	+
B. pneumon. Friedl.	-	B. tetani	+
B. rhinoskleromat.	-	C. diphtheriae	+
B. dysent. Flexner	-	Diphtheroid bac.	+
B. dysent. Y.	-	Aktinomyzes	+
B. vulgare	+/-	B. Timothee	+
Oidium albicans	+	Paramaezien	

With the exception of oidium albicans, on one side there are only gram-negative, on the other side only gram-positive micro-organisms. The former developed well and abundantly in the mesohemin plate whereas the latter do not develop at all. Among these are very important pathogenic bacteria such as staphylococci, streptococci, diphtheria, anthrax, etc. Unfortunately, I have not yet been able to test all important species of bacteria, specifically gram-negative micrococci and b. tuberculosis. However, since actinomyces and b. timothy are inhibited, I do not doubt that the tuberculosis group can also be listed in the second column. The growth-inhibiting and/or killing action of mesohemin is often very intensive but appears to differ for the respective individual species. Even after dilution of mesohemin to 1:500,000, b. anthracis still shows definite inhibition of growth. For pneumococci, inhibition was observed only up to a dilution of 1:64,000. However, it is possible that here the blood protein (which was not present in the experiments with b. anthracis) interfered, similar to the familiar mercuric-chloride experiments of Behring.

We know that we must distinguish between growth-inhibiting and killing or antiseptic action. The antiseptic property of a medium is demonstrated in permitting it to act for a given length of time on the bacteria in the liquid substratum and then inoculating an agar culture with a small amount of the liquid containing the bacteria. The inhibiting action of the slight amount of the medium transferred simultaneously must be eliminated through controls. I thus found that staphylococci are killed almost completely within 75 minutes by a mesohemin solution in a dilution of 1:2000 but that the growth-inhibiting action on staphylococcus extends at least as far as a dilution of 1:128,000. At a dilution of 1:10,000, I was not longer able to observe any difference for staphylococci in the "antiseptic" experiment whereas a difference from the control could be noted for b. anthracis even at this dilution and during a period of exposure of 80 minutes. The killing action on pneumococci was also demonstrated in the animal experiment. 2 mice were infected with pneumococci previously treated with a 1-% mesohemin solution. The animals survived whereas the control mice inoculated with non-treated pneumococci died in about 30 hours. Specific experiments on the killing of spores are not yet available.

The antibacterial action of mesohemin on the respective groups consequently is rather important, exceeds in some species of bacteria the action of carbolic acid, the soluble silver salts, etc. and approaches that of mercuric chloride. However, the elective character as well as the insufficient action of the substance on gram-negative species is shown by the fact that, for example, typhus germs grown in mesohemin solutions are not at all inhibited in their motility. The experiments illustrate simultaneously the enormous biological difference produced by even such slight changes as the addition of 4 hydrogen atoms in the molecule of mesohemin in contrast to heme or the absence of iron as against hematoporphyrin (cf. note below).

(NOTE: Hematin or $C_{34}H_{32}N_4O_4FeOH$ (hemin or $C_{34}H_{32}N_4O_4FeCl$) through alkaline reduction of Hematin; mesohematin or $C_{34}H_{36}N_4O_4FeOH$ (mesohemin or $C_{34}H_{36}N_4O_4FeCl$) through acid reaction from hematin; hematoporphyrin or $C_{34}H_{36}N_4O_6$ from the latter through reduction: mesoporphyrin or $C_{34}H_{36}N_4O_4$. However, hematin is not completely without damaging action on some species of bacteria. I was able to achieve complete inhibition of growth for b. anthracis and megatheium at a dilution of 1:300.

We already knew that the vitality of paramecia is quickly inhibited by hematoporphyrin. We did not investigate the reactions of heme, bilirubin and mesohemin. Whereas the paramecia still swam gayly about 24 hours after the addition of heme and bilirubin to the hanging drop, their motility ceases at the latest 15 minutes after adding mesohemin (exactly as for hematoporphyrin), i.e. they obviously die. By contrast, phagocytosis of leucocytes (human) in vitro and of guinea pigs in vivo (peritoneal liquor) remains apparently completely unaffected on the basis of my present findings.

We might ask whether light does not also play a role in the killing of microorganisms by mesochemin as in the sensitization of the hematoporphyrin animal. However, corresponding experiments with staphylococci in the dark chamber show the same bactericidal action also in the dark.

Of considerable significance for my investigations appeared to me to be the observations of Churchman published in 1912 (Journal of Experimental Medicine, Vol. 16, p. 221) who demonstrated that gentian violet (the familiar gram-staining medium) as well as other pararosanilines such as parafuchsin, dahlia, methyl violet but also rosaniline in high dilutions, had a growth-inhibiting action on gram-positive bacteria whereas they completely failed to affect the gram-negative bacteria, i.e. a very similar behavior that consequently is perhaps a characteristic also of other types of pigment. The gram-resistance of bacilli is probably based, especially in view of the investigations of Brudny and Eisenberg, on their permeability by the pigments mentioned whereas the gram-negative bacteria are impermeable to them. The pigments accordingly have no toxic action on the gram-negative bacteria because they cannot penetrate the bacterial body. In this or in a similar manner, we might also explain the elective influence on gram-positive bacteria by mesohemin.

However, the action of this hemoglobin derivative is certainly of greater importance than that of the pararosanilines. It is not completely excluded that mesohemin exists temporarily in the body through reduction of heme which is even more probable for heme and hematoporphyrin. It is not impossible that the bactericidal action of the body fluids is perhaps related in part to the content of mesohemin and possibly also of heme and similar substances.

But why is the bactericidal action of mesoporphyrin so intensive and that of heme and hematoporphyrin practically non-existing? According to a law promulgated by Overton, a substance is absorbed from an aqueous medium by the bacterial cell as much more quickly as it is readily soluble in lipoids, i.e. in the lipoid border layers of the bacterial body. I was able to demonstrate by a simple experiment that, in the slightly alkaline pigment solutions utilized by me, the salt of mesohemin has a closer relation to lipoid substances than the compounds of heme and hematoporphyrin contained in the respective solutions, i.e. when extracting the slightly alkaline aqueous solutions with ether, mesohemin is definitely absorbed into the latter but heme and hematoporphyrin are not. It is probable that this is at least a partial explanation for the difference in action.

The thought of utilizing the considerable destructive and/or inhibiting action of mesohemin on bacteria through chemotherapy is obvious. Such experiments are already under way but without having produced any reliable results so far. It was shown unfortunately that mesohemin in high doses constitutes a toxin for liver, spleen and the kidneys. In

small amounts -- still sufficiently active as shown by experimentation in vitro and in consideration of (animal) body weight, mesohemin was tolerated by rabbits without any apparent damage. We should perhaps consider a primarily local utilization because of the low susceptibility of the medium to resorption and its intensive action on cocci.

FIGURE APPENDIX

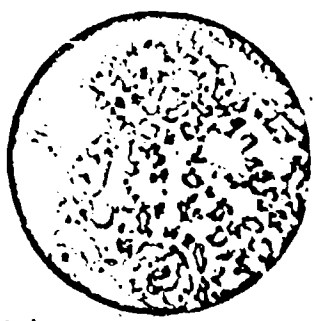

Fig. 1. Hemalin 1 : 2000 (Control)

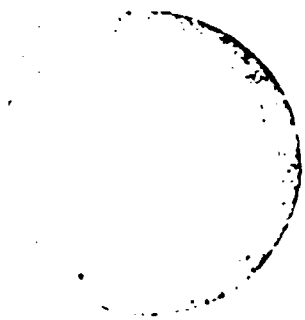

Fig. 2. Mesohaemalin 1 : 8000.

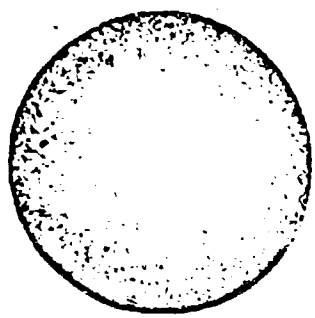

Fig. 3. Mesohaemalin 1 : 16000.

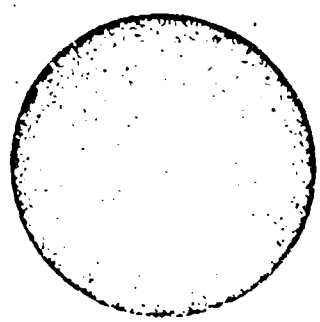

Fig. 4. Mesohaemalin 1 : 32000.

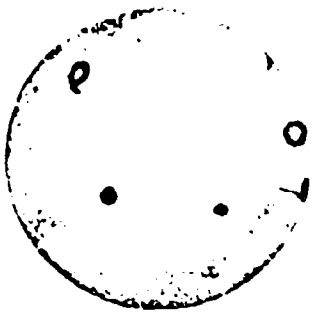

Fig. 5. Mesohaematin 1 : 64000.

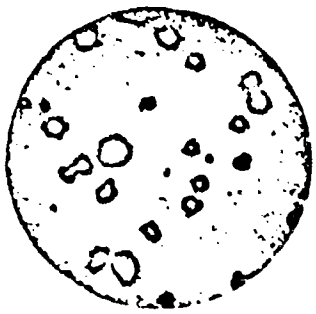

Fig. 6. Mesohaematin 1 : 128000.

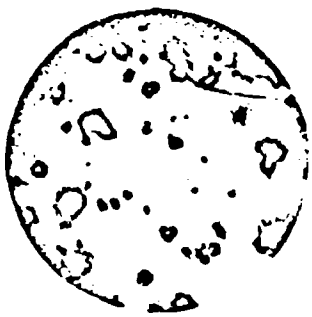

Fig. 7. Mesohaematin 1 : 256000.

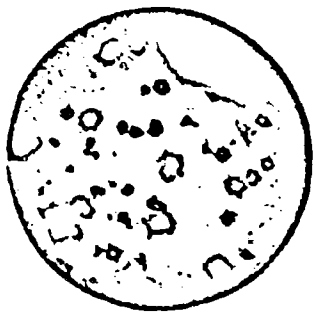

Fig. 8. Mesohaematin 1 : 512000.

The figures represent photographs of agar plates (magnification objective 2, ocular 4, Leitz). After preparing 10 ccm of liquified agar, cooled to 44° C, containing heme or mesohemin in the dilutions as indicated below the figures, these were inoculated with 3 loops from a culture of b. anthracis incubated for 24 hours and then poured into Petri dishes. The photos were made after incubation at 37° C for 24 hours.